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C/EBPβ Is a Negative Regulator of Human Papillomavirus Type 11 in Keratinocytes

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We have evaluated the impact of the CCAAT enhancer-binding protein (C/EBP) transcription factors on human papillomavirus type 11 (HPV11). C/EBPβ is in nuclei of cultured foreskin keratinocytes and binds its consensus sequence in HPV11 DNA. We have used the novel approach of depleting the availability of C/EBPs in vivo using nuclease-resistant oligomers containing C/EBP DNA binding sites. In cultured foreskin keratinocytes containing replicating HPV11 DNA, levels of both HPV11 transcripts and HPV DNA increase after treatment with oligomers containing the C/EBPβ DNA binding motif. These results indicate that C/EBPβ is a repressor for HPV11 in keratinocytes.

The human papillomaviruses (HPVs) infect basal cells of stratifying squamous epithelia. In the basal cells, HPV transcription and DNA replication are at a very low level. It is only in suprabasal cells that viral transcripts and DNA replication can become amplified (10, 52). We know little about cellular factors that repress or enhance HPV expression in keratinocytes. Most likely, transcription factors that program differentiation are responsible for the alternate expression, at least in part. Transcription factors known as CCAAT enhancer-binding proteins (C/EBPs) program differentiation in a wide variety of tissues, and HPV DNAs contain C/EBP recognition sequences. Therefore, we wanted to know if C/EBPs have an impact on HPV transcription and replication.

Briefly, the C/EBPs are a family of DNA-binding proteins belonging to bZIP proteins that form homodimers and heterodimers, which in turn bind C/EBP DNA binding motifs and CCAAT sequences. Separate genes encode different C/EBPs, and different initiation start sites generate multiple isoforms of individual C/EBPs. C/EBPs are an integral part of the regulation of tissue-specific terminal differentiation, e.g., myelomonocytic cells (13, 23, 39, 47), hepatocytes (22, 37), and adipocytes (11, 30, 31, 53). The abundance and ratios of different C/EBPs (or individual C/EBP isoforms) determine their activities as transcription factors. C/EBPβ, the primary focus in this study, has been variously called LAP, IL6-DBP, CRP2, ACP/EBP, and NF-IL6 (36). In adipocyte differentiation, C/EBPβ is active only in preadipocytes (11, 17) and plays a key role in monocyte/macrophage differentiation (13, 23, 47). Other C/EBPs or a truncated C/EBPβ known as LIP (14) will inactivate C/EBPβ.

C/EBPs do interact with HPVs. We have determined that proteins extracted from laryngeal papillomas bind consensus motifs for C/EBPα and C/EBPβ in an HPV type 11 (HPV11) DNA fragment (7, 8) and that recombinant C/EBPα expressed in bacteria binds these sequences (8). Others have shown that C/EBPs bind HPV16 DNA (28, 48) and bovine PV4 DNA (35). C/EBPβ, made in vitro, binds C/EBP consensus sequences in HPV16 DNA and also AP-1 and NF-1 DNA binding motifs (28). Overexpression of C/EBPβ down regulates the HPV16 E6/E7 promoter (28). In this report, we show that C/EBPβ is present in cultured human foreskin keratinocytes (HFKs) and binds HPV11 sequences. Increased expression and replication of HPV11 DNA occur when we deplete C/EBPβ in vivo.

Western blot (immunoblot) analysis of proteins prepared from HFKs (45) used polyclonal antibodies to C/EBPβ, C/EBPα, C/EBPδ, or involucrin. The preparation of nuclease-resistant oligomers and their possible use in vivo was described by Clusel et al. (12). The electrophoretic mobility shift assay was essentially as described previously by us (7, 8), and supershifts were as described by McCaffery and Jackson (35). Stable transfection of HPV11 DNA in HFKs was as described by Mungal et al. (38). Reverse transcription-PCR was done as described by Maran et al. (34). Southern or slot blot procedures were used to analyze HPV DNA. An NF-IL6 expression vector with a cytomegalovirus promoter was the generous gift of S. Akira, Osaka, Japan, and the control vector was the same plasmid with the NF-IL6 sequences removed. The CaptureTec system (Invitrogen) was used to select cells in cotransfections that were cotransfected with the pHook vector. The pHook vector expresses a single-chain antibody against a specific hapten on the surface of transfectants, and such cells can be isolated by binding to hapten-coated magnetic beads.

C/EBPβ was clearly present in HFKs (Fig. 1). A protein in nuclear extracts reacted with C/EBPβ antibodies and was the expected size of full-length C/EBPβ. Both C/EBPβ and C/EBPα were detectable in cytoplasmic extracts and were probably dimers that were not easily denatured (55). C/EBPδ was not detectable. Involucrin was detectable only in cytoplasmic extracts, indicating the integrity of nuclear and cytoplasmic fractions and also that some keratinocyte differentiation was detectable.

Depleting the availability of C/EBPβ in vivo is a means to determine its effect on HPV11. Evaluation of the function of the C/EBP family of transcription factors poses problems, because homo- and heterodimers of the different C/EBPs have different effects on gene expression. Moreover, C/EBPs can interact with other transcription factors, interfering with their binding (21), competing for binding sites (6, 18, 19, 28, 32, 42), synergistically transactivating genes (26, 27, 29, 40, 49, 51), or

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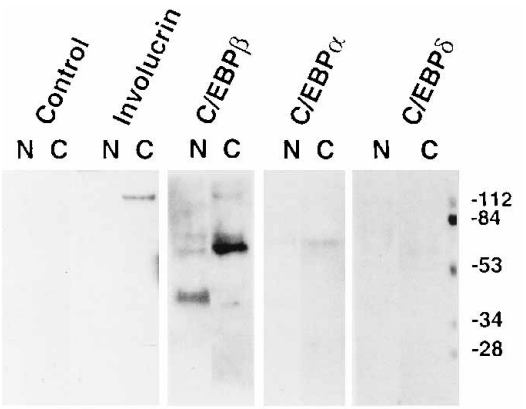

FIG. 1. C/EBPβ is present in cultured keratinocytes. Western analysis used nuclear (N) and cytoplasmic (C) protein extracts (15) from HFKs separated by electrophoresis in sodium dodecyl sulfate–12% polyacrylamide gels and reacted with normal rabbit serum (control), rabbit polyclonal antibodies made to human C/EBPβ amino acids 199 to 345, rat C/EBPα amino acids 253 to 265, rat C/EBPδ amino acids 253 to 265 (Santa Cruz Biotechnologies), and involucrin (Biomedical Technologies Inc.). The second antibody was peroxidase-labelled goat antibody to rabbit immunoglobulin G (Kirkegaard & Perry Laboratories, Inc.), and development was with enhanced chemiluminescence reagents (Amersham). Molecular weights (in thousands) of protein markers are indicated.

regulating genes that regulate other transcription factors (1, 2, 20, 23, 25, 27, 43, 46). Our approach was to use nuclease-resistant oligomers containing the C/EBPβ binding motif to deplete the availability of this protein in in vivo assays. Essentially, such oligomers have closed ends and are hence called dumbbells. The closed ends make them resistant to exonucleases. The desired binding motif is in the double-stranded region of the molecule as shown in Fig. 2A for the binding sites of C/EBPβ. We wanted to ensure that C/EBPβ in HFKs bound to HPV11 DNA and that the dumbbell was an effective competitor for such DNA-protein binding in vitro. The electrophoretic mobility shift assay used nuclear extracts of HFKs, synthetic double-stranded oligomers containing the HPV11 DNA C/EBPβ binding site at nucleotides (nt) 7451 to 7459, and antibodies to C/EBPβ (Fig. 2B). A single shift resulted from the binding, and a supershift occurred when antibody was present. Antibody to C/EBPα did not result in a supershift. Both unlabelled oligomer and the C/EBPβ dumbbells severely reduced binding.

To confirm that our HFKs take up small oligomers as has been reported by others (41), we used nonspecific dumbbells with a single fluorescent tag (Fig. 2C). With a dumbbell concentration of 50 nM in the culture medium, nuclei of most cells were brightly fluorescent at 4 h after the addition of dumbbells and fluorescent nuclei were still detectable 72 h later. In studies, new medium containing dumbbells was added to cells every 3 days. Dumbbells were not toxic to the cells. No difference in the percentages of cells able to exclude trypan blue occurred between cells that were untreated and cells that were treated with 50 nM C/EBPα, C/EBPβ, or nonspecific dumbbells, even after 14 days of treatment (data not shown).

C/EBPβ is a repressor of HPV11 transcription and replication. We then evaluated the effect of dumbbells on HPV11 transcription and replication in HFKs after stable transfection of HPV11 DNA. These nonimmortalized cells replicate episomal HPV11 DNA (38), and HPV11 transcripts are detectable only by reverse transcription-PCR (34). HPV11 transcripts increased after treatment with the C/EBPβ dumbbells (Fig. 3A to C). E6/E7 transcripts were evaluated by reverse transcription-PCR, using primer sets nt 514 to 533 and nt 4076 to 4166, which would exclude transcripts starting from downstream promoters. We determined when amplifications of E6/E7, β-actin, or human acidic ribosomal phosphoprotein PO (36B4) were severely limited or abolished by using decreasing amounts of cDNA. Clearly, HPV PCR products were detectable in cells treated with the C/EBPβ dumbbells at a dilution higher than that in untreated (control) cells (Fig. 3A) or cells treated with a dumbbell with a mutant C/EBPβ binding motif (Fig. 3B). In a time course, an increase in the level of HPV11 transcripts was detectable at the earliest time point, 24 h after the addition of the C/EBPβ dumbbells. We believe the predominant HPV transcript is 1,100 bp (Fig. 3), and the 1,900-bp transcripts (Fig. 3C) are those described by Maran et al. (34) for similar cells with the same primer set. The smaller HPV transcript, seen only in cells treated with the C/EBPβ dumbbell, has not been previously described. With these primers, the unspliced PCR product is 3,652 bp (not shown). Our result complements the results of Kyo et al. (28), who found that transfection of an expression vector under the strong cytomegalovirus promoter repressed transcription of HPV16 in the CaSki cell line. Therefore, C/EBPβ appears to be a general repressor of HPV transcription.

C/EBPβ altered the amount of HPV DNA in HFKs containing HPV11 DNA cells. A C/EBPβ expression vector caused a decrease in the level of HPV11 DNA, whereas treatment with C/EBPβ dumbbells increased the HPV DNA level (Fig. 4A). A C/EBPβ (NF-IL6) expression vector was transfected together with the pHook vector. After 48 h, transfected cells (binding to hapten-coated beads recognizing antibody coded by pHook) and nontransfected cells (not binding to beads) were allowed to grow for 7 days, because the average doubling time of cultured keratinocytes is approximately 72 h (4). Less HPV DNA was present in the transfected cells than in the nontransfected cells. Conversely, the HFKs treated with dumbbells contained more HPV DNA than did untreated cells. We expanded these experiments (Fig. 4B) to show that C/EBPβ caused a decrease in the level of HPV11 DNA whereas the vector without C/EBPβ sequences did not. Moreover, the dumbbells abrogated the decrease. Hapten-selected transfected cells were treated with dumbbells for 7 days. HPV and β-actin DNAs were measured by slot blot analysis and by comparison to standards by densitometry. In this experiment, the HFKs transfected with the C/EBPβ expression vector contained only 25% as much HPV11 DNA as cells transfected with an empty cytomegalovirus expression vector. C/EBPβ dumbbells abrogated the decrease.

In further analysis of treatment of HPV-transfected HFKs with dumbbells, the increase in the level of HPV11 DNA was dependent on the concentration of C/EBPβ dumbbells reaching a near maximum level of 50 nM in all experiments (Fig. 4C). However, the consistent increase was variable and was different in different HFK cells and in different passages. The increase with 50 nM C/EBPβ dumbbells was 2-, 3-, 4-, 17-, or 55-fold with different transfected cells or passages. The amount of HPV11 DNA did not increase after treatment with nonspecific dumbbells or with C/EBPα dumbbells. The result shown (55×) used cells at passage 8, while more modest increases were at lower passages 3 and 4. Results were similar by Southern analysis (Fig. 4D) using genomic DNA and by hybridization with HPV11 DNA. The amounts of HPV DNA increased approximately twofold with cells at passage 2 and fivefold with cells at passage 5 (quantitated by densitometry). The different passages contained different amounts of HPV DNA. Variability between experiments was not surprising, because cultured HFKs were from different individuals and contained mixed populations of keratinocytes (4). The amount of HPV11 DNA in infected cells changes, usually decreases, as cells are passaged (6a, 13a, 16) and in HFKs infected with HPV1 (44). Despite the variability, treatment with C/EBPβ dumbbells always resulted in increased amounts of HPV DNA. We can

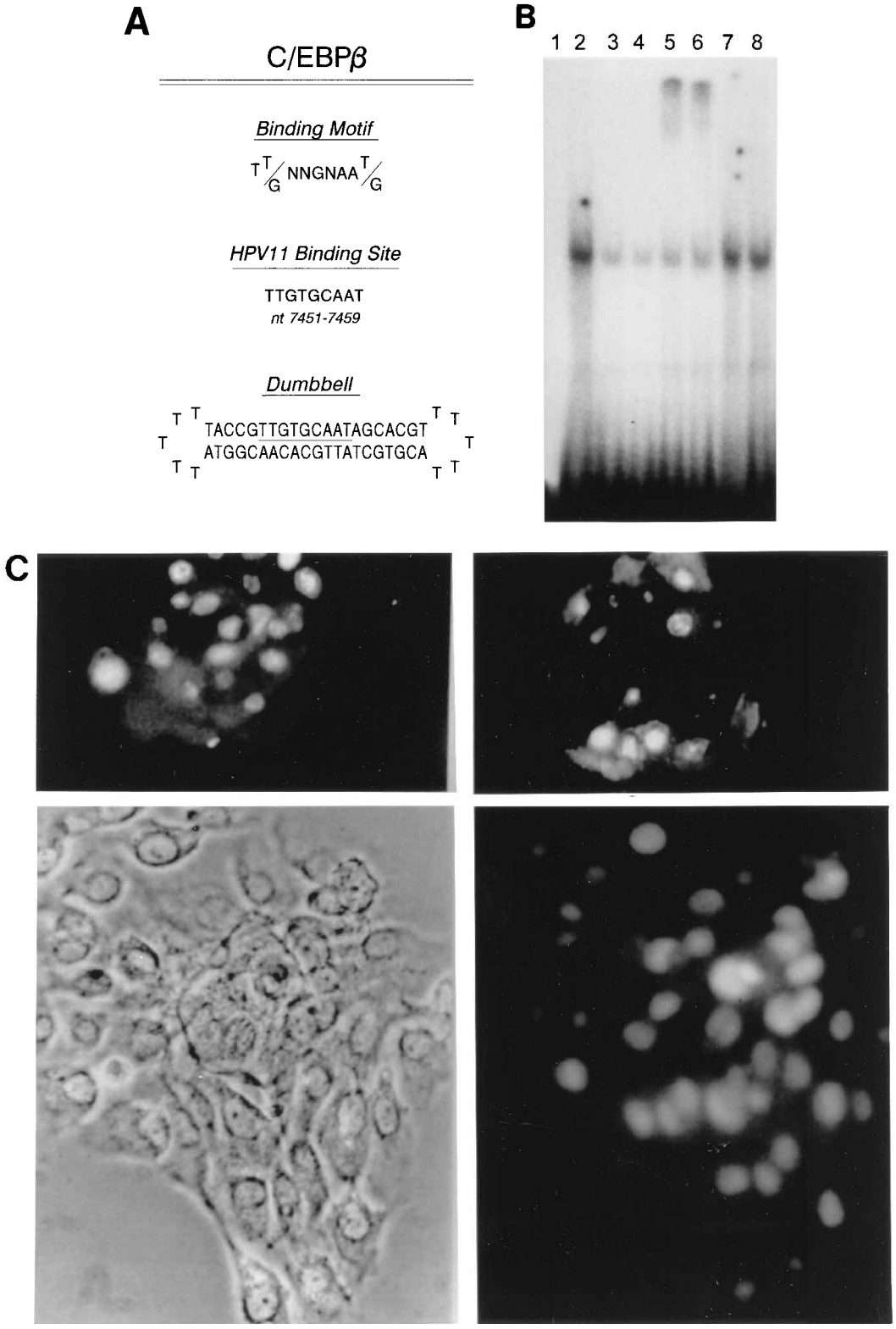

FIG. 2. Competency of dumbbells. Dumbbells compete in in vitro DNA-protein binding, and dumbbells are taken up by cells and the target nucleus. (A) Sequence and structure of the dumbbell for C/EBPβ. The preparation of dumbbells (12) was linear DNA synthesis that included a 5′ phosphorylated end (DNA network synthesis; University of Medicine and Dentistry of New Jersey, Piscataway, N.J.) and then annealing and ligation with T4 ligase. (B) Binding of keratinocyte proteins with a C/EBPβ binding motif in HPV11 and a supershift with antibody to C/EBPβ in an electrophoretic mobility shift assay. Each binding reaction mixture contained 3.3 μg of keratinocyte nuclear extract (15) and 0.1 pmol of a ^{32}P end-labelled double-sided oligomer, TACCGTTGTGCAATAGCACGTCGTGTGC, in gel shift buffer (Promega) containing poly(dI-dC). Electrophoresis was in 6% polyacrylamide gels. Lane 1 is labelled oligomer only. Lanes 2 to 8 show the results of binding reactions with the following additions: 12 pmol of unlabelled oligomer (lane 3); 12 pmol of C/EBPβ dumbbell (lane 4); 0.5 and 2 μl of polyclonal antibody made to rat C/EBPβ amino acids 258 to 276 (lanes 5 and 6, respectively); and 0.5 and 2 μl of rabbit polyclonal antibody to rat C/EBPα amino acids 253 to 265 (lanes 7 and 8, respectively). Antibodies were from Santa Cruz Biotechnologies. (C) HFKs after the addition of medium containing 50 nM of a nonspecific dumbbell with a single fluorescent tag (DNA network synthesis). The times after the addition of dumbbells were 24 h (upper left photograph), 72 h (upper right photograph), and 48 h (the two lower photographs). The same cells are shown in the lower photographs. Magnification, ×64 (upper photographs) and ×80 (lower photographs).

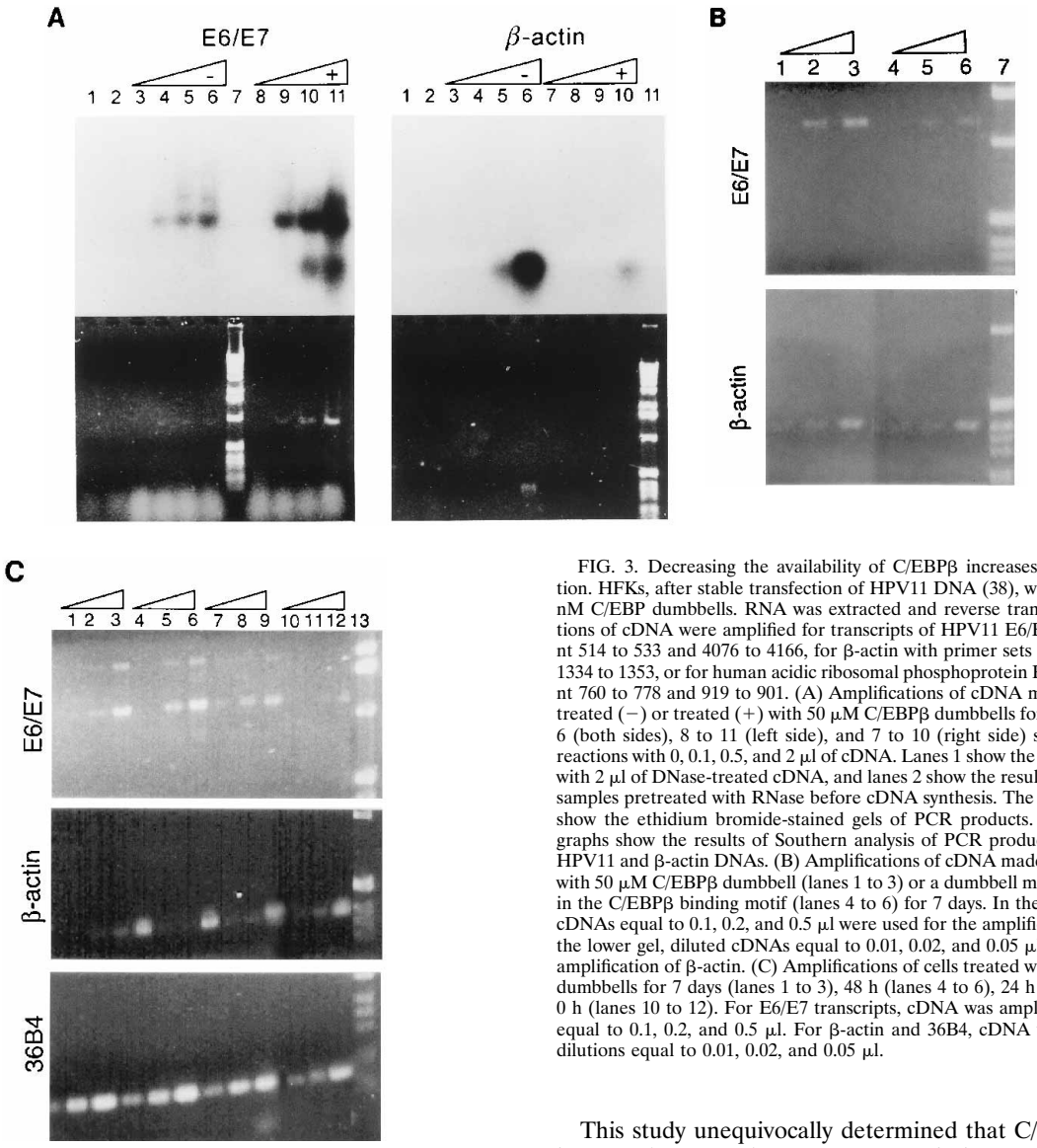

FIG. 3. Decreasing the availability of C/EBPβ increases HPV11 transcription. HFKs, after stable transfection of HPV11 DNA (38), were treated with 50 nM C/EBP dumbbells. RNA was extracted and reverse transcribed (34). Dilutions of cDNA were amplified for transcripts of HPV11 E6/E7 with primer sets nt 514 to 533 and 4076 to 4166, for β-actin with primer sets nt 985 to 1004 and 1334 to 1353, or for human acidic ribosomal phosphoprotein PO with primer sets nt 760 to 778 and 919 to 901. (A) Amplifications of cDNA made from cells not treated (−) or treated (+) with 50 μM C/EBPβ dumbbells for 7 days. Lanes 3 to 6 (both sides), 8 to 11 (left side), and 7 to 10 (right side) show the results of reactions with 0, 0.1, 0.5, and 2 μl of cDNA. Lanes 1 show the results of reactions with 2 μl of DNase-treated cDNA, and lanes 2 show the results of reactions with samples pretreated with RNase before cDNA synthesis. The lower photographs show the ethidium bromide-stained gels of PCR products. The upper photographs show the results of Southern analysis of PCR products hybridized with HPV11 and β-actin DNAs. (B) Amplifications of cDNA made from cells treated with 50 μM C/EBPβ dumbbell (lanes 1 to 3) or a dumbbell made with mutations in the C/EBPβ binding motif (lanes 4 to 6) for 7 days. In the upper gel, diluted cDNAs equal to 0.1, 0.2, and 0.5 μl were used for the amplification of E6/E7. In the lower gel, diluted cDNAs equal to 0.01, 0.02, and 0.05 μl were used for the amplification of β-actin. (C) Amplifications of cells treated with 50 μM C/EBPβ dumbbells for 7 days (lanes 1 to 3), 48 h (lanes 4 to 6), 24 h (lanes 7 to 9), and 0 h (lanes 10 to 12). For E6/E7 transcripts, cDNA was amplified with dilutions equal to 0.1, 0.2, and 0.5 μl. For β-actin and 36B4, cDNA was amplified with dilutions equal to 0.01, 0.02, and 0.05 μl.

conclude that increasing the level of C/EBPβ results in decreased HPV DNA replication whereas decreasing C/EBPβ levels increases HPV replication.

This study clearly establishes a link between C/EBPβ and levels of expression and replication of the HPVs.

Using a novel approach, we were able to show that depleting C/EBPβ in HFKs resulted in increased transcription and replication of HPV11 DNA. This approach of treating cells with nuclease-resistant oligomers containing the C/EBPβ DNA binding motif has many parallels to treating cells with antisense oligomers but also permits depletion of possible heterodimers with other transcription factors. Our approach thus has many advantages, because the competition for C/EBPβ occurs (i) in vivo, (ii) in the context of all natural regulatory elements and open reading frames of HPV11, (iii) in the context of other transcription factors acting on HPV11 DNA, and (iv) in the background of genes that may be regulated by C/EBPβ and that in turn regulate HPV11. In essence, this method accounts for the total effect of making C/EBPβ unavailable as a DNA-binding protein in cells.

This study unequivocally determined that C/EBPβ, at least in part, down regulates HPV transcription and replication in HFKs. C/EBPβ's effect on HPV may be a direct interaction with HPV DNA, indirect effects as outlined above, or both. Clearly, C/EBPβ can interact with HPV DNA. Decreasing the availability of C/EBPβ, as was done in the experiments described above, relieved repression. In nature, decreasing the availability of a C/EBPβ occurs because other C/EBPs are expressed or move to the nucleus and complex C/EBPβ. Presumably, modulation of any C/EBP could change the availability of C/EBPβ. The list of factors that are known to induce (or repress) C/EBPs is extensive. They include epidermal growth factor (37), bacterial lipopolysaccharide (1–3), interleukin 1 (1, 26), interleukin 6 (1, 20), phorbol esters (23), retinoids (20), calcium (9, 54), glucocorticoids (33), and tumor necrosis factor (1, 26, 50). Many of these modulate epithelial growth and differentiation. C/EBPs not only form dimers with each other and their truncated isoforms (14, 24, 36) but also form dimers with other bZIP proteins, such as Fos or Jun (21), another mode for changing their availability. It is entirely possible that other C/EBPs could be activators for HPV11, apart from C/EBPβ being a repressor. Many families, e.g., Myc-Max-Mad, of transcription factors use the process of one member being

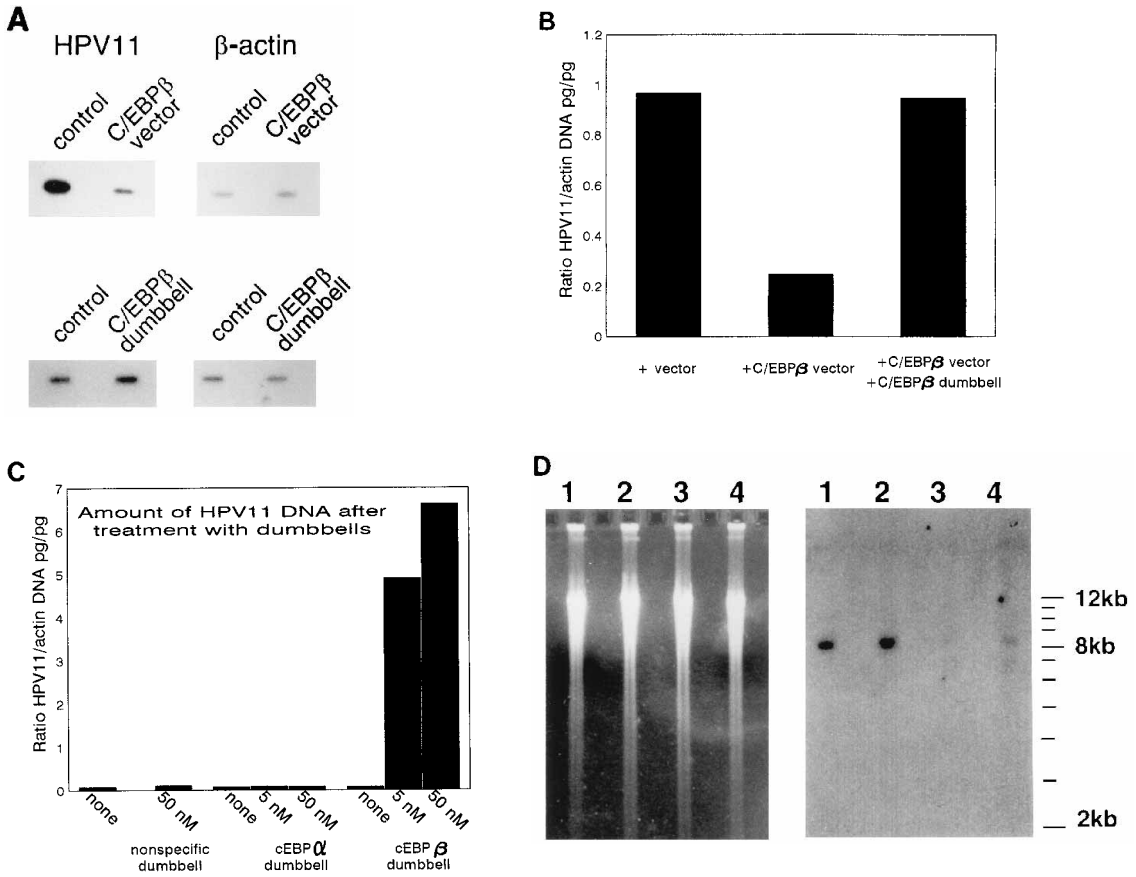

FIG. 4. Decreasing the availability of C/EBPβ increases HPV11 replication. Increasing the availability of C/EBPβ decreases HPV11 replication. (A) HFKs, after stable transfection with HPV11 DNA, were cotransfected with a C/EBPβ expression vector (gift of S. Akira) and the pHook Capture-Tec vector (Invitrogen). After 48 h, cells were selected with phOx-coated magnetic beads, the hapten being directed against the antibody encoded by pHook. Nonselected cells (control) and selected cells were grown for 7 days, and DNAs were extracted and hybridized sequentially with ^{32}P-labelled HPV11 DNA and β-actin DNA by slot blot analysis (upper blot). Similarly, HFKs, stably transfected with HPV11 DNA, were treated with 50 μM C/EBPβ dumbbells for 7 days, and DNA was compared with a parallel culture (control) of the same untreated cells by slot blot analysis (lower blot). (B) HFKs, stably transfected with HPV11 DNA, were cotransfected with a C/EBPβ expression vector (26) or an empty vector without C/EBPβ coding sequences together with pHook-1 plasmid DNA. After 48 h, transfected cells were selected as described for panel A. Selected cells were untreated or treated with 50 nM C/EBPβ dumbbells for 7 days, and DNAs were extracted. By a slot blot analysis, the amounts of HPV11 and β-actin DNAs were quantitated by densitometry and by comparison to HPV11 and β-actin DNA standards after sequential hybridization with ^{32}P-labelled HPV11 and β-actin DNAs. (C) HFKs, stably transfected with HPV11 DNA, were untreated or treated with nonspecific C/EBPα (containing the consensus binding motif at nt 7429 to 7436 in HPV11) or C/EBPβ dumbbells for 7 days. Total DNA was extracted. By a slot blot analysis, the amounts of HPV11 and β-actin DNAs were quantitated as described for panel B. (D) Southern analysis of DNAs from different passages of HFKs that were stably transfected with HPV11 DNA. Passage 2 HFKs (lanes 1 and 2) and passage 5 HFKs (lanes 3 and 4) were untreated (lanes 1 and 3) or treated (lanes 2 and 4) with 50 nM C/EBPβ dumbbells. In each lane, 14 μg of genomic DNA was cut with *Bam*HI restriction enzyme and electrophoresed (left gel), transferred to a membrane (Southern), and hybridized to a ^{32}P-labelled HPV11 DNA fragment (right gel). DNA size markers are indicated.

an activator with another member acting as a repressor, such that the relative amount of each member determines the net effect (5). If C/EBPβ represses and other C/EBPs enhance HPV11 expression, the expectation might be a continuum of increased expression and replication if C/EBPs change with keratinocyte differentiation, as is the case in other tissues.

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